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The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites

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PEA3 is a transcription factor which binds to the polyoma virus enhancer and whose activity is regulated by the expression of a number of oncogenes. We show here that PEA3 also binds specifically to the collagenase and *fos* cellular promoters. On the collagenase promoter, PEA3 acts synergistically with AP-1 to achieve maximum levels of transcription activation by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), and non-nuclear oncoproteins, thereby defining a TPA- and oncogene-responsive unit (TORU). From a comparative study of the collagenase TORU and the analogous polyoma virus TORU, we conclude that both the binding affinity of the PEA3 motif and the spacing between PEA3 and AP-1 modulate transcription activation induced by oncogene expression.
Key words: AP1/collagenase/fos/oncogenes/PEA3

Introduction

In the last few years, considerable progress has been made in the identification of transcription factors whose activity is regulated by oncogene expression, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) or serum. One of these factors, AP-1, has been shown to regulate genes which are highly expressed in transformed cells, such as stromelysin, c-*fos* and collagenase (reviewed in Herrlich and Ponta, 1989; Imler and Wasylyk, 1989). Other factors include NF-κB (Lenardo and Baltimore, 1989), serum response factor (SRF; Schröter *et al.*, 1987; Treisman, 1987), p62 (Shaw *et al.*, 1989a) and PEA3 (Wasylyk *et al.*, 1989). SRF binds to a dyad symmetry sequence, the serum responsive element (SRE), located between −320 and −299 of the human c-*fos* promoter. Mutations in the SRE abolish induction of c-*fos* transcription by serum, TPA and several growth factors (Greenberg *et al.*, 1987; Gilman, 1988; Sheng *et al.*, 1988). Another factor, named p62, which binds immediately upstream of the SRF, is required in addition to SRF for induction of c-*fos* transcription by serum (Shaw *et al.*, 1989a). We have previously reported that PEA3, a factor that binds to the polyoma (Py) virus enhancer (nucleotides 5107–5113; Martin *et al.*, 1988), is induced by serum, TPA and the non-nuclear oncogenes v-*src*, Py middle T, Ha-*ras*, v-*mos* and v-*raf* (Wasylyk *et al.*, 1989).

In the present work, we demonstrate that PEA3 binds to the collagenase promoter and that it acts synergistically with AP-1 to achieve maximal induction of transcription by TPA and several oncogenes. In addition we show that PEA3 binds to a sequence in the c-*fos* promoter required for serum induction of transcription (Shaw *et al.*, 1989a), suggesting that PEA3 could be one of the mediators of the response of the c-*fos* promoter to serum components.

Results

PEA3 binds to collagenase and c-fos promoters

By searching through the EMBL databank, we have identified putative PEA3 binding-sites in several gene promoters (Wasylyk *et al.*, 1989) including the human c-*fos* proto-oncogene (nucleotides −321 to −314) and human collagenase (nucleotides −89 to −82). The PEA3-like motifs in the collagenase and *fos* genes (referred to as PEA3 COL and PEA3 FOS), however, are not identical to the PEA3 motif of the Py enhancer (referred to as PEA3 Py); they differ by 2 and 1 bp respectively (see Figure 1A, lines 1–3). To investigate the binding affinities of these motifs, we used complementary oligonucleotides containing the human collagenase and c-*fos* PEA3-like sites and the PEA3 Py motif as probes in gel-shift experiments. Both the PEA3 COL and PEA3 FOS probes form complexes that comigrate with the PEA3 Py complex (Figure 2, lanes 1–3). In addition, the PEA3 COL and PEA3 FOS probes are competed by an excess of cold PEA3 Py (Figure 2, lanes 5–7 and 12–14, respectively), but not with a mutated PEA3 motif (data not shown) demonstrating that PEA3 can bind to the collagenase and *fos* promoters. To determine the relative affinities of these PEA3 binding sites, we studied the competition between the complexes formed with the PEA3 COL and PEA3 FOS probes and increasing concentrations of either cold PEA3 COL or PEA3 FOS (Figure 2, lanes 8–10 for PEA3 COL and lanes 15–17 for PEA3 FOS). Both PEA3 COL and PEA3 FOS are poorer competitors than PEA3 Py (compare lanes 8–10 with lanes 5–7 and lanes 15–17 with lanes 12–14, in Figure 2) indicating that PEA3 has a higher affinity for the Py motif. By comparing the competitor concentrations needed to reduce binding to 50%, we estimate that the PEA3 Py sequence has a five-fold higher affinity than the PEA3 COL and PEA3 FOS motifs.

PEA3 molecular weight determination

To characterize further the nature of the proteins involved in complex formation with the PEA3 Py, PEA3 FOS and PEA3 COL probes, we performed preparative gel-retardation assays similar to the one shown in Figure 2, excised the specific complexes, treated them with formaldehyde in order to crosslink protein to DNA and fractionated the crosslinked complexes by denaturing electrophoresis (SDS–PAGE). In Figure 3 it is shown that only one comigrating band is present for each probe, with an apparent molecular weight of 65 kd (this method slightly overestimates the molecular weight because of the presence of the crosslinked 17-mer oligonucleotide). The relative intensities of the crosslinked species are similar to those of

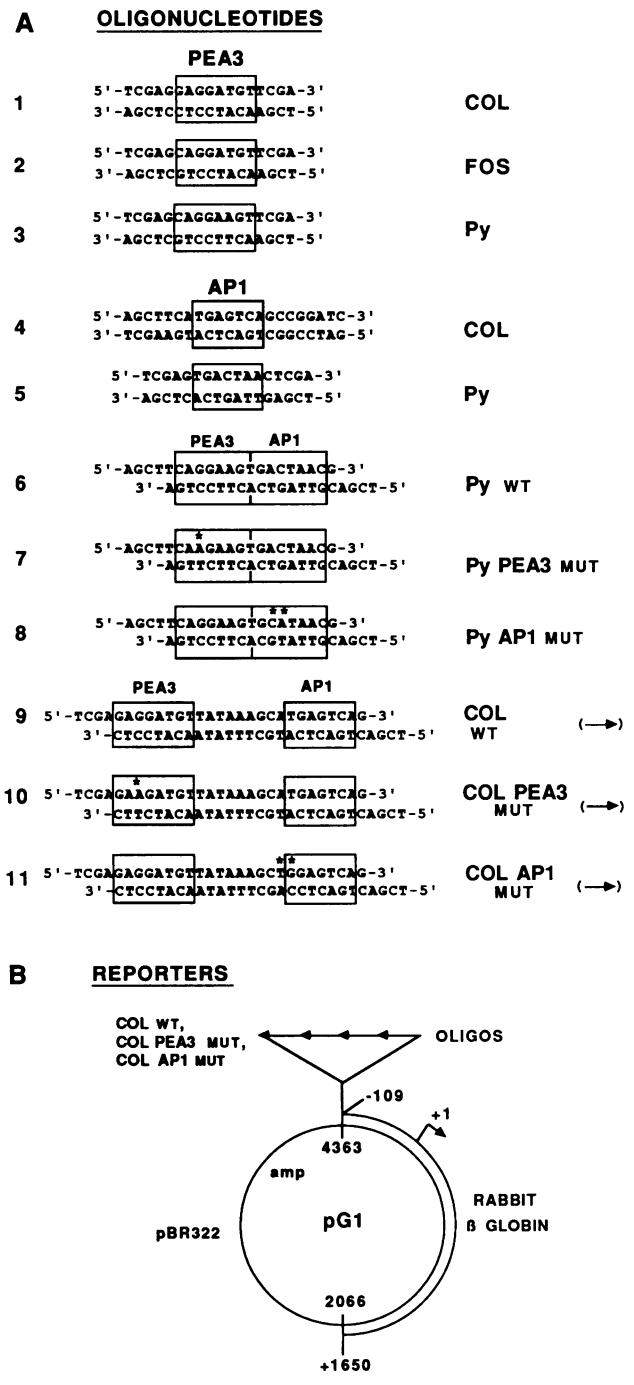

Fig. 1. Sequence of oligonucleotides (**A**) and structure of reporter recombinants (**B**). Oligonucleotides 1–8 were used in gel-retardation experiments; oligonucleotides 9–11 were used both as probes in gel retardation experiments and to construct the reporter recombinants shown in B. Py PEA3 mut and COL PEA3 mut have point mutations in their sequences which inhibit PEA3 binding (Martin *et al.*, 1988). Py AP-1 mut and COL AP-1 mut have mutations in the AP-1 motif which inhibit binding of their cognate factor (our unpublished observations and Angel *et al.*, 1988; respectively). PEA3 and AP-1 motifs are boxed and mutated bases are indicated by a star. The reporters contain four head-to-tail copies of oligonucleotides (→) upstream of the β-globin promoter of pG1.

the retarded complexes (compare Figure 2, lanes 1–3 with Figure 3), suggesting that the efficiency of crosslinking was similar for each DNA–protein complex. These results support the hypothesis that the same protein binds to the three different PEA3 motifs.

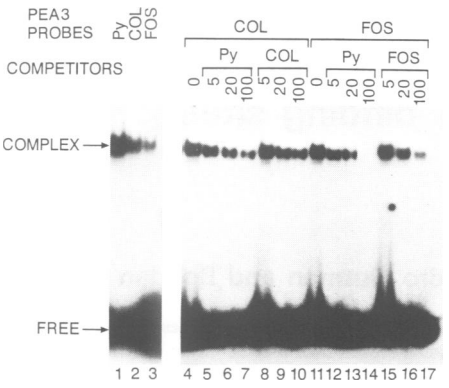

Fig. 2. PEA3 binding to collagenase and c-*fos* PEA3-like motifs. Whole cell extracts (10 μg protein) of HeLa cells were preincubated with the indicated molar excess (5, 20, 100) of PEA3 competitor oligonucleotides for 15 min at 4°C before the addition of the indicated PEA3 probes. After a further 10 min at 25°C, the protein–DNA complexes were resolved on 5% acrylamide gels. Specific complexes and free unretarded probe are shown.

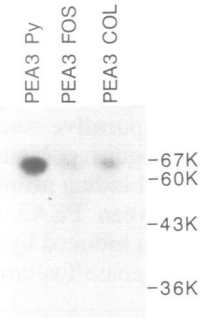

Fig. 3. PEA3 molecular weight determination by formaldehyde crosslinking. PEA3 Py, PEA3 FOS and PEA3 COL labelled probes were each incubated with 200 μg HeLa whole cell extracts as described in Materials and methods. The retarded complexes, with similar relative intensities to those shown in Figure 2, lanes 1–3, were crosslinked, mixed with sample buffer and resolved on a 10% denaturing polyacrylamide gel (SDS–PAGE). The molecular weight of marker proteins is indicated.

PEA3 acts synergistically with AP-1 in the collagenase promoter to achieve a maximum level of transcription activation by non-nuclear oncoproteins

The collagenase promoter contains an AP-1 site a few nucleotides downstream from the PEA3 site (Figure 1A, line 9). It has been reported that this AP-1 site is necessary for transcription activation by TPA and some oncoproteins (Angel *et al.*, 1987a; Schöntal *et al.*, 1988). We decided to investigate whether PEA3 plays a role in the induction of transcription of the collagenase promoter by oncogenes. We synthesized oligonucleotides containing a minimal collagenase promoter and encompassing the PEA3 and AP-1 motifs (COL wt; Figure 1A, line 9). We also introduced point mutations into the PEA3 (COL PEA3 mut; Figure 1A, line 10) or AP-1 motifs (COL AP-1 mut; Figure 1A, line 11). We inserted these oligonucleotides as tetramers into the expression vector pG1 (Figure 1B) and measured the ability of these sequences to stimulate transcription from the β-globin promoter. The reporter recombinants, together with an internal control plasmid, were cotransfected with different oncogene expression vectors into LMTK$^-$ fibroblasts and, after 48 h of culture in low serum (0.05% fetal calf serum),

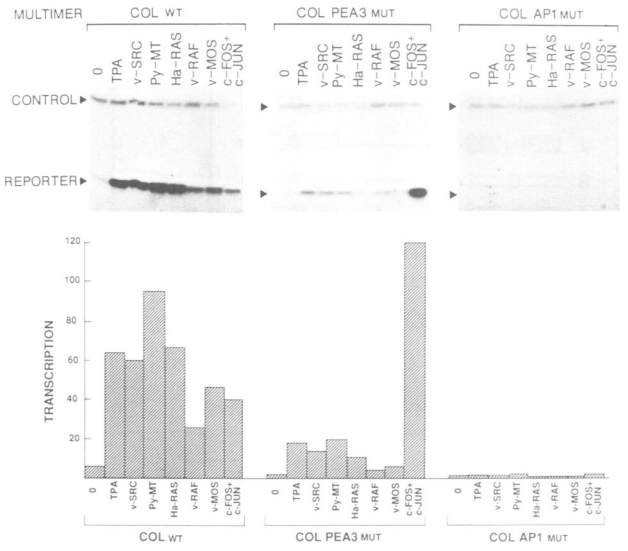

Fig. 4. Effect of TPA and oncogene expression on COL wt, COL PEA3 mut and COL AP-1 mut multimer activity. Subconfluent LMTK$^-$ fibroblasts (9 cm plates) were transfected with 1.5 μg of the indicated reporters, 0.5 μg of the internal control plasmid (pBCB × 2) and 5 μg of expression vectors for the indicated oncogenes. TPA, when indicated, was added 24 h later at 100 μg/ml. After 40 h in culture in low serum (0.05% FCS), total RNA was analysed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporters (Reporter bands) and the conalbumin promoter of the control recombinants (Control bands). The ratio of specific transcription from the multimer containing reporters to that of the parent vector is represented by a histogram. Transcription values are the average from several experiments and are corrected for variation in pG1 expression with oncogenes. pG1 transcription level is considered equal to 1.

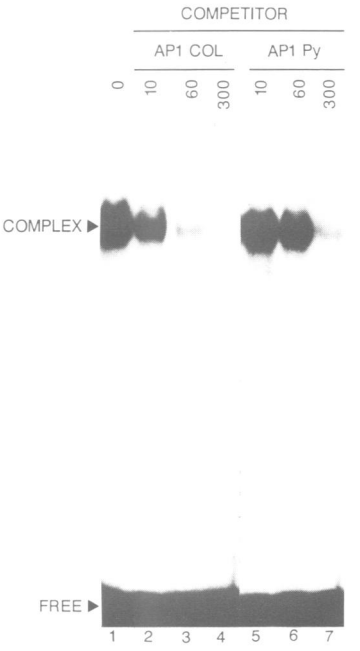

Fig. 5. Comparative binding affinities of AP-1 COL and AP-1 Py. AP-1 COL labelled probe was incubated with HeLa whole cell extracts (10 μg protein) in gel-retardation experiments. Preincubation with increasing concentrations of competitors was done when indicated. Specific complexes and free, unretarded probe bands are shown.

total cellular RNA was extracted. Quantitative S1 nuclease mapping was used to measure the amount of specific RNA initiated from the reporter and control recombinant promoters (see Reporter and Control bands in Figure 4). The transfections were repeated at least three times, with two different preparations of DNA, to ensure reproducibility of the results.

Figure 4 shows the basal level of globin transcription (in the absence of inducer) and the levels induced by TPA or by cotransfection with expression vectors for v-*src*, Py middle-T, Ha-*ras*, v-*raf*, v-*mos* and c-*fos* + c-*jun*. Multimer activity is corrected for variations in the expression of the parent vector pG1 and is measured as the transcription ratio between multimer-containing plasmids and pG1 (transcription from pG1 = 1). COL wt basal level of transcription is 5 times higher than that of pG1 and induced COL wt levels vary from 25 to 90, depending on the inducing agent. A point mutation in the PEA3 motif reduces the basal level of transcription by 50% and the TPA- and oncogene-induced levels to 15−27% of wild type (COL PEA3 mut in Figure 4), with the exception of c-*fos* + c-*jun*. With these two oncogenes, the PEA3 mutation, in contrast, increases their induction abilities three-fold. This result agrees with previous observations that showed that PEA3 activity is independent of *fos* expression (Wasylyk *et al.*, 1989). The mutation in the AP-1 motif completely abolishes the inducibility of the minimal collagenase promoter (Figure 4, COL AP-1 mut) with TPA and with all the oncogenes tested. In this way, we define a TPA- and oncogene-responsive unit (TORU) in the collagenase promoter, composed of two different but interacting elements, PEA3 and AP-1.

Collagenase and Py virus AP-1 motifs have different binding affinities

The collagenase TORU resembles the Py virus TORU in its composition (see Figure 1A, lines 6 and 9), but both the spacing and the sequence of the PEA3 and AP-1 motifs are different. We analysed binding affinities of collagenase and Py AP-1 motifs (referred to as AP-1 COL and AP-1 Py) by gel-shift experiments. We used an oligonucleotide probe containing the AP-1 COL motif and we studied the competition between it and an excess of either AP-1 COL (Figure 5, lanes 2−4) or AP-1 Py (Figure 5, lanes 5−7). As shown, AP-1 COL is a better competitor than AP-1 Py. For example, a 10-fold excess of AP-1 COL cold competitor reduces binding to ~50% (compare lanes 1 and 2 in Figure 5) but a 60-fold excess of AP-1 Py competitor reduces binding by only 30% (compare lanes 1 and 6 in Figure 5), indicating that the affinity of the AP-1 collagenase motif is more than six times higher than that of the Py virus AP-1 motif.

PEA3 binding affinity is independent of AP-1 binding

As we showed recently for the Py virus enhancer (Wasylyk *et al.*, 1989) and as we observe here for the collagenase promoter, PEA3 and AP-1 have synergistic effects in the activation of transcription by oncogenes. We considered the possibility that AP-1 binding could increase the affinity of PEA3 for its binding site. To test this hypothesis, we did gel-retardation assays with the PEA3 Py and PEA3 COL motifs as probes and we studied the competition between them and wild-type and mutated versions (of either PEA3 or AP-1 motifs) of the corresponding TORUs (see Figure 1A, lines 6−8 and lines 9−11 for the sequences of the Py and collagenase competitors, respectively). There are no differences in the competitor efficiencies whether the

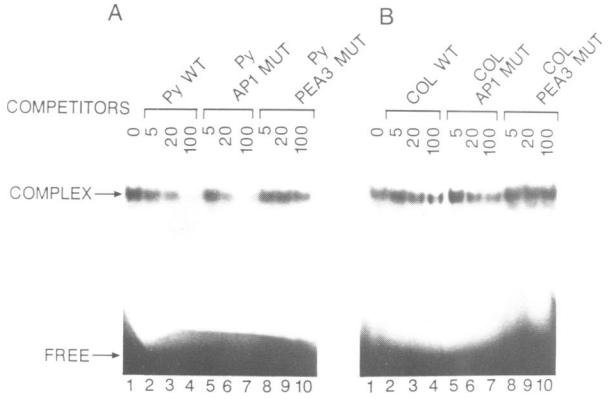

Fig. 6. Effect of AP-1 binding on PEA3 binding affinity. Gel-retardation experiments were performed by incubating HeLa whole cell extracts (10 μg protein) with PEA3 Py (**A**) and PEA3 COL (**B**) labelled probes. Preincubation with increasing concentrations of competitors was done where indicated. Specific complexes and free, unretarded probes are shown.

AP-1 binding site is mutated or not (compare lanes 2–4 with 5–7 in Figure 6A and B for the PEA3 Py and PEA3 COL probes, respectively) suggesting that the affinity of PEA3 for its binding site is the same irrespective of whether AP-1 is bound to the DNA. In contrast, a point mutation in the PEA3 motif completely abolished its ability to compete (lanes 8–10 in Figure 6A and B for PEA3 Py and PEA3 COL probes, respectively), showing that competition was specific.

Both the binding affinity of PEA3 and the spacing between PEA3 and AP-1 motifs modulate the activity of collagenase and Py virus TORUs

Considering the results reported above, we can decribe the collagenase TORU as being composed of a 'low' affinity PEA3 motif and a 'high' affinity AP-1 motif separated by 9 nucleotides. The Py virus TORU has, in contrast, a 'high' affinity PEA3 element immediately adjacent to a 'low' affinity AP-1 element (see Figure 7, lines 1 and 8 for a schematic description of these two TORUs). In order to understand the relative importance of binding affinities and spacing of PEA3 and AP-1 motifs in determining the inducibility of the TORUs described above, we constructed eight different recombinants. They are depicted in Figure 7 (their sequences are presented in Materials and methods). Four constructions contain the PEA3 and AP-1 elements with the spacing which they have in the collagenase promoter (lines 1–4). The four others have adjacent PEA3 and AP-1 motifs as in the Py virus enhancer (lines 5–8). Within each group, four relevant combinations of PEA3 and AP-1 elements are represented: PEA3 COL/AP-1 COL (lines 1 and 5); PEA3 Py/AP-1 COL (lines 2 and 6); PEA3 COL/AP-1 Py (lines 3 and 7); PEA3 Py/AP-1 Py (lines 4 and 8). These oligonucleotides were inserted as monomers in the expression vector pG1 and recombinants were transfected in LMTK$^-$ fibroblasts, together with a control plasmid, and with or without an expression vector coding for v-src protein. Transcription levels were measured by S1 nuclease mapping as described above and the results are shown in Figure 7. The activities of the two naturally occurring TORUs are rather different; the induced level of the collagenase TORU is relatively low compared to the Py TORU (about 0.4 times lower; compare lines 1 and 8,

TPA AND ONCOGENE RESPONSIVE UNIT		TRANSCRIPTION	
	PEA3 MOTIF / AP1 MOTIF	BASAL	INDUCED
1	C / C	3	6
2	Py / C	4	12
3	C / Py	2	6
4	Py / Py	2	7
	PEA3 MOTIF / AP1 MOTIF		
5	C / C	3	9
6	Py / C	5	16
7	C / Py	2	6
8	Py / Py	5	16

Fig. 7. Basal and v-src induced transcriptional activity of natural and chimeric TPA- and oncogene-responsive units. Eight different recombinants were constructed by insertion of complementary oligonucleotides (whose sequences are presented in Materials and methods) as monomers in the expression vector pG1. Its schematic structure is shown. PEA3 and AP-1 motifs are indicated with their corresponding spacing; Py virus and collagenase motifs are shown by dark and white arrows, respectively. The relative binding affinity of PEA3 and AP-1 motifs is represented by thin or thick arrows for low and high affinity binding sites, respectively. Subconfluent LMTK$^-$ fibroblasts (9 cm plates) were transfected with 1.5 μg of the indicated reporters, 0.5 μg of the internal control plasmid (pBCB × 2) and 5 μg of v-*src* expression vectors when indicated. After 40 h in culture in low serum (0.05% FCS), total RNA was analysed by quantitative S1 nuclease mapping for RNA initiated from the β-globin promoter of the reporters and the conalbumin promoter of the control recombinants. Transcription levels were calculated as the ratio of specific transcription from the insert containing reporters to that of the parent vector and they represent the average from several experiments (pG1 transcription level is considered equal to 1).

respectively). The collagenase TORU can be classified as a 'low responder' compared with the chimeric TORUs (presented in Figure 7). In contrast, the Py TORU can be considered as a 'high responder', in this context. What changes would be needed to increase the responsiveness of a TORU? Replacing the 'weak' PEA3 COL element by the 'strong' PEA3 Py element significantly increases the activity of the TORU (compare lines 1 and 2) but this is not enough to equal Py TORU activity. Substitution of the AP-1 COL 'strong' motif in the collagenase TORU, on the contrary, does not produce detectable changes in transcription levels (compare lines 3 and 4 with line 1). Reducing the spacing between PEA3 and AP-1 motifs in the collagenase TORU (mimicking the Py TORU organization) only slightly increases the transcriptional activity (compare lines 1 and 5). To attain maximum levels of transcription it is necessary to make two changes in the collagenase TORU: to increase the affinity of the PEA3 motif, and to reduce spacing (compare lines 1 and 6). This chimeric TORU is not affected by the substitution of the AP-1 COL 'strong' element by the AP-1 Py 'weak' motif (giving the natural Py TORU) (compare lines 6 and 8). In contrast, replacing the PEA3 Py motif by the PEA3 COL element decreases transcription levels considerably (compare lines 6 and 7).

In conclusion, two main factors seem to be important in modulating the level of activity of these TORUs: the spacing, and the binding affinity of the PEA3 motif. In contrast, even though AP-1 is necessary to confer inducibility on the

TORU, as was shown above, a decrease of more than six-fold in the binding affinity of the AP-1 motif does not seem to alter significantly the transcriptional activity of the TORUs.

Discussion

PEA3 binds to collagenase and c-fos promoters

We show that the transcription factor PEA3 which binds to the Py virus enhancer also binds to similar motifs in the collagenase and c-*fos* promoters, but with somewhat lower affinity. The *fos* motif has been shown by Shaw *et al.* (1989a) to be required, along with SRE, for serum induction of *fos* promoter activity. This suggests that PEA3 could be involved somehow in the serum response. Shaw *et al.* (1989a) identified a factor, p62, which binds to this motif, but only when associated with the serum response factor (SRF). A third factor, p62/MAPF1, also binds to the same element (Ryan *et al.*, 1989; Walsh, 1989). Further studies are required to establish the relationship between these factors and their roles in *fos* promoter activity.

Interactions between PEA3 and AP-1 modulate the activity of collagenase and Py virus TORUs

Angel *et al.* (1987a and b) have reported that the collagenase gene promoter contains a TPA-responsive element that binds the AP-1 factor(s). The same element mediates transcription activation by oncogenes (Schöntal *et al.*, 1988; this work). Experiments with deletion mutants of the collagenase promoter suggested the existence of additional inducible elements upstream of the AP-1 binding site (Angel *et al.*, 1987a; Schöntal *et al.*, 1988). We show here that both the PEA3 and AP-1 motifs participate in the response of the collagenase promoter to TPA and v-*src*, Py-mT, Ha-*ras*, v-*raf* and v-*mos* oncogenes, thereby delimiting a TORU. AP-1 is essential to confer oncogene inducibility on this domain, but maximum levels of transcription activation can only be attained with the cooperation of PEA3.

Gel-retardation experiments suggest that synergistic effects between PEA3 and AP-1 are not the result of cooperation at the DNA binding level, because PEA3 binding affinity is the same irrespective of whether AP-1 is bound. Similar observations have been reported for other eukaryotic transcription factors. To explain these observations, Ptashne (1988) proposed that two eukaryotic activators can work synergistically not because they touch each other, but rather because they simultaneously touch a third (target) protein (promiscuous cooperativity). However, the particular mechanism by which PEA3 and AP-1 cooperate remain to be investigated. Interestingly, the spacing between the PEA3 and AP-1 motifs seems to be an important factor in determining transcriptional activity, as revealed by analysis of v-src induced transcription of collagenase and Py TORUs, compared to that of chimeric constructions containing different combinations of Py and collagenase motifs. Maximum levels of activity were only seen with recombinants that had adjacent PEA3 and AP-1 elements (as in the Py enhancer). A high affinity PEA3 motif is also necessary to achieve high levels of induced transcription, showing that PEA3 binding can modulate transcription activation of TORUs. In contrast, a considerable decrease in the binding affinity of the AP-1 motif can be tolerated without loss of activity. This could be explained by the increase of intracellular levels of AP-1 due to v-*src* expression. In these conditions, the amount of AP-1 could be sufficiently high that differences in the affinities of the AP-1 motifs do not limit their ability to mediate activation of transcription. In contrast, in the induced conditions, PEA3 levels could be limiting, so that the relative binding affinities of the PEA3 motifs would reflect their ability to mediate transcription.

TORUs are nuclear targets for regulation of transcription by oncogenes

There is increasing evidence that different transcription factors can mediate gene activation by TPA and oncogenes. In addition to the AP-1 family, other factors that could be included in this group are NF-$\varkappa$B, serum response factor, p62 and PEA3. It has been proposed (Schöntal *et al.*, 1988; Wasylyk *et al.*, 1988; Herrlich and Ponta, 1989; Imler and Wasylyk, 1989) that extracellular signals can activate a cascade of membrane and cytoplasmic oncoproteins which in turn regulate gene expression through transcription factors. The same oncogenes and TPA activate AP-1 (Angel *et al.*, 1987a, Imler *et al.*, 1988; Schöntal *et al.*, 1988; Wasylyk *et al.*, 1988), NF-$\varkappa$B (Sen and Baltimore, 1986; Lenardo and Baltimore, 1989; Imler *et al.*, manuscript in preparation) and PEA3 (Wasylyk *et al.*, 1989, this work) suggesting that these factors are the nuclear targets of a common pathway. It is interesting that at least one of these targets, AP-1, is composed of fos and jun oncoproteins. This raises the question of whether other targets could themselves turn out to be oncogenes. Recently two other nuclear oncoproteins, myb (Biedenkapp *et al.*, 1988) and myc (Iguchi-Ariga *et al.*, 1988; Ariga *et al.*, 1989) have been shown to bind to specific DNA sequences. In addition the *myb* motif can activate transcription from heterologous promoters (Ness *et al.*, 1989; Nishina *et al.*, 1989; Weston and Bishop, 1989). Other oncogenes that are potential transcription factors are *ski* (Nomura *et al.*, 1989) and the *ets* gene family (Boulukos *et al.*, 1989). It is not yet known if non-nuclear oncoproteins can also regulate gene expression through the *myc* and *myb* motifs. Further research is necessary to determinate how many pathways exist for oncogene transcription activation of specific genes.

Binding sites for oncogene inducible factors have been considered mainly as isolated responsive elements. Previous work (Wasylyk *et al.*, 1989) and the present report show that binding sites for two of these factors can associate to form what we call a TORU. Different types of TORU could exist: (i) simple, formed from only one autonomous responsive element, e.g. AP-1 or NF-$\varkappa$B binding sites; (ii) composite, formed from two or more interacting elements. The Py virus enhancer and the collagenase promoter are examples of this second type, each composed of a PEA3 and a AP-1 binding site. Another member, with a different organization, could be the c-*fos* promoter in which several different factors seem to interact: p62, SRF, AP-1 and possibly PEA3 and p62/MAPF1. Both the AP-1 binding site and the SRE can mediate TPA activation of the *fos* promoter, but there are no synergistic effects between these two elements suggesting that they may not be able to bind protein simultaneously (Fisch *et al.*, 1989). SRE can mediate induction if SRF binding occurs in the presence of p62, but it can also mediate repression through a fos−SRF interaction (Leung and Miyamoto, 1989; Lucibello *et al.*, 1989; König *et al.*, 1989; Shaw *et al.*, 1989b). Other types of TORU remain to be characterized.

Considering the modular organization of enhancers and promoters (Dynam, 1989), we propose that TORUs are specialized inducible enhancer modules which play a double role. They interact with other elements to determine basal transcriptional activity of the enhancer. In addition, they mediate high level variations of gene expression in response to oncogene stimulation and they could thus participate in the pathways leading to cellular transformation.

Materials and methods

Extract preparation and gel mobility shift assays

Whole cell extracts were a gift from J.M.Egly. Gel mobility shift assays were performed by incubating 10 μg of cell extract with 3 μg of poly(dI.dC)−poly(dI.dC) and excess 5′ ^{32}P-labelled oligonucleotides, as described by Martin *et al.* (1988). Non-labelled competitors were added, where indicated, at the same time as the poly(dI.dC)−poly(dI.dC). The samples were fractionated by electrophoresis through a 5% polyacrylamide gel, run in 45 mM Tris base, 45 mM boric acid 5 mM EDTA.

DNA−protein crosslinking

Gel-retardation experiments were performed as described above but on a preparative scale (200 μg of whole cell extract in a 100 μl volume reaction). Fractionation of the samples was done by electrophoresis through a 1% low melting point agarose gel, run in 90 mM Tris base, 90 mM boric acid, 10 mM EDTA. Autoradiography was performed at 4°C (1 h) and specific complexes were excised. The gel slices were then incubated for 4 h with 500 μl of 2% formaldehyde and subsequently washed two times with electrophoresis buffer, followed by heating for 5 min at 65°C. The volume was measured and SDS-sample buffer was added, followed by fractionation on a 10% denaturing polyacrylamide gel electrophoresis (SDS−PAGE).

Transfections and S1 nuclease analysis

The COL wt, COL PEA3 mut and COL AP-1 mut recombinants were constructed by inserting four head-to-tail copies of complementary oligonucleotides (Figure 1A, lines 9−11) into pG1. Recombinants shown schematically in Figure 7 were constructed by inserting the following oligonucleotides (annealed previously with their corresponding complementary oligonucleotides) in the *Hin*dIII /*Xho*I sites of pG1 expression vector:

1. 5′-AGCTTGAGGATGTTATAAAGCATGAGTCAGG-3′
2. 5′-AGCTTCAGGAAGTTATAAAGCATGAGTCAGG-3′
3. 5′-AGCTTGAGGATGTTATAAAGCATGACTAACG-3′
4. 5′-AGCTTCAGGAAGTTATAAAGCATGACTAACG-3′
5. 5′AGCTTGAGGATGTGAGTCAGG-3′
6. 5′-AGCTTCAGGAAGTGAGTCAGG-3′
7. 5′-AGCTTGAGGATGTGACTAACG-3′
8. 5′-AGCTTCAGGAAGTGACTAACG-3′

The structure of oncogene expression vectors, transfections and quantitative S1 nuclease analysis have been described previously (Wasylyk *et al.*, 1987; Imler *et al.*, 1988; Wasylyk *et al.*, 1988).

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References

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahnsdorf,H.J., Jonat,C., Herrlich,P. and Karin,M. (1987a) *Cell*, **49**, 729−739.

Angel,P., Baumann,I., Stein,B., Delius,H., Rahmsdorf,H.J. and Herrlich,P. (1987b) *Mol. Cell. Biol.*, **7**, 2256−2266.

Angel,P., Hattori,K., Smeal,T. and Karin,M. (1988) *Cell*, **55**, 875−885.

Ariga,H., Imamura,Y. and Iguchi-Ariga,S. (1989) *EMBO J.*, **8**, 4273−4279.

Biedenkapp,H., Borgmeyer,U., Sippel,A.E. and Klempnauer,K.-H. (1988) *Nature*, **335**, 835−837.

Boulukos,K.E., Pognonec,P., Rabault,B., Begue,A., and Ghysdael,J. (1989) *Mol. Cell. Biol.*, **9**, 5718−5721.

Dynam,W.S. (1989) *Cell*, **58**, 1−4.

Fisch,T.H., Prywes,R. and Roeder,R.G. (1989) *Mol. Cell. Biol.*, **9**, 1327−1331.

Gilman,M.Z. (1988) *Genes Dev.*, **2**, 394−402.

Greenberg,M.E., Siegfried,Z. and Ziff,E.B. (1987) *Mol. Cell. Biol.*, **7**, 1217−1225.

Herrlich,P. and Ponta,H. (1989) *Trends Genet.*, **5**, 112−116.

Iguchi-Ariga,S.M.M., Okazaki,T., Itani,M., Ogata,M., Sato,Y. and Ariga,H. (1988) *EMBO J.*, **7**, 3135−3142.

Imler,J.L., Schatz,C., Wasylyk,C., Chatton,B. and Wasylyk,B. (1988) *Nature*, **332**, 275−278.

Imler,J.L. and Wasylyk,B. (1989) In Heath,J.K. (ed.), *Progress in Growth Factor Research*. Pergamon Press, Vol. 1, pp. 69−77.

König,H., Ponta,H., Rahmsdorf,Y., Büscher,M., Schöntal,A., Rahmsdorf, H.J. and Herrlich,P. (1989) *EMBO J.*, **8**, 2559−2566.

Lenardo,M.J. and Baltimore,D. (1989) *Cell*, **58**, 227−229.

Leung,S. and Miyamoto,N.G. (1989) *Nucleic Acids Res.*, **17**, 1177−1195.

Lucibello,F.C., Lowas,C., Neuberg,M. and Müller,R. (1989) *Cell*, **59**, 999−1007.

Martin,M.E., Piette,J., Yaniv,M., Tang,W.J. and Folk,W.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5839−5843.

Ness,S.A., Marknell,A. and Graf,T. (1989) *Cell*, **59**, 1115−1125.

Nishina,Y., Nagagoshi,H., Imamoto,F., Gonda,Th.J. and Ishi,S. (1989) *Nucleic Acids Res.*, **17**, 107−117.

Nomura,N., Sasamoto,S., Ishi,S., Date,T., Matsui,M. and Ryotaro,I. (1989) *Nucleic Acids Res.*, **17**, 5489−5500.

Ptashne,M. (1988) *Nature*, **335**, 683−689.

Ryan,W.A.,Jr., Franza,B.R.,Jr. and Gilman,M.Z. (1989) *EMBO J.*, **8**, 1785−1792.

Schöntal,A., Herrlich,P., Rahmsdorf,H.J. and Ponta,H. (1988) *Cell*, **54**, 325−334.

Schröter,H., Shaw,P.E. and Nordheim,A. (1987) *Nucleic Acids Res.*, **15**, 10145−10158.

Sen,R. and Baltimore,D. (1986) *Cell*, **47**, 921−928.

Shaw,P.E., Schröter,H. and Nordheim,A. (1989a) *Cell*, **56**, 563−572.

Shaw,P.E., Frasch,S. and Nordheim,A. (1989b) *EMBO J.*, **8**, 2567−2574.

Sheng,M., Dougan,S.T., McFadden,G. and Greenberg,M.E. (1988) *Mol. Cell. Biol.*, **8**, 2787−2796.

Treisman,R. (1987) *EMBO J.*, **6**, 2711−2717.

Walsh,K. (1989) *Mol. Cell. Biol.*, **9**, 2191−2201.

Wasylyk,C., Imler,J.L., Perez-Mutul,J. and Wasylyk,B. (1987) *Cell*, **48**, 525−534.

Wasylyk,C., Imler,J.L. and Wasylyk,B. (1988) *EMBO J.*, **7**, 2475−2483.

Wasylyk,C., Flores,P., Gutman,A. and Wasylyk,B. (1989) *EMBO J.*, **8**, 3371−3378.

Weston,K. and Bishop,M.J. (1989) *Cell*, **58**, 85−93.

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